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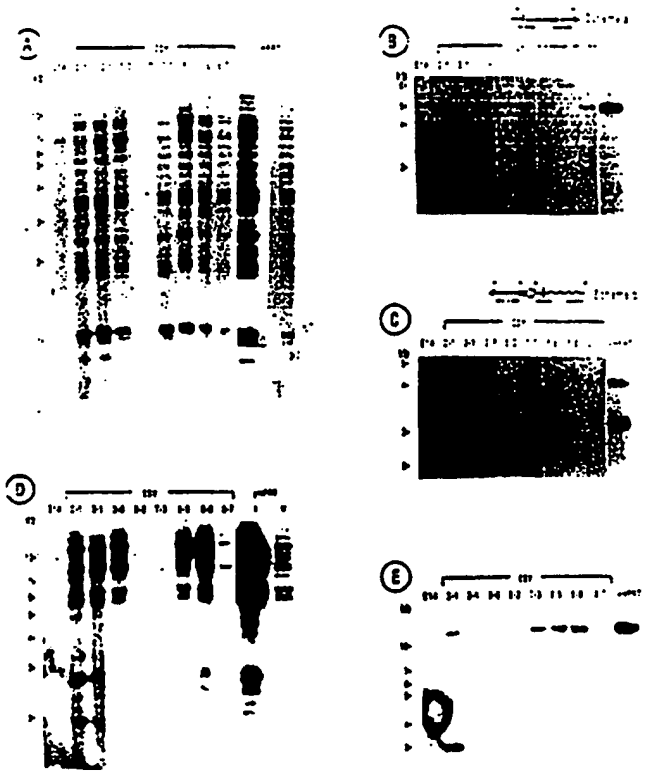
(54) Title: METHOD TO IMPROVE SCREENING EFFICIENCY IN FUSED CELLS

(57) Abstract

A method for improving the efficiency of screening fused cells for the presence of desired genes is disclosed. The method involves providing the desired gene with a marker that overcomes the sensitivity of an immortalizing cell fusion partner to certain medium conditions. The method is illustrated by the inclusion of a marker on immunoglobulin genes present in B cells.



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METHOD TO IMPROVE SCREENING EFFICIENCY IN FUSED CELLS

This is a continuation-in-part application of U.S. Serial No. 08/234,145 filed 28 April 1994 which is a continuation-in-part of U.S. Serial No. 08/112,848 filed 27 August 1993 which is a continuation-in-part of U.S. Serial No. 08/031,801 filed 15 March 1993 which is a continuation-in-part of U.S. Serial No. 07/919,297 filed 24 July 1992 which is a continuation-in-part of U.S. Serial No. 07/610,515 filed 8 November 1990 which is a continuation-in-part of U.S. Serial No. 07/466,008 filed 12 January 1990. The contents of these applications are incorporated herein by reference.

Technical Field

The invention is directed a method to increase the percentage of fused cell colonies which contain a desired genomic feature. More specifically, the invention concerns providing a marker proximal to the desired genomic feature which will permit an immortalizing cell to grow under conditions to which it would otherwise be sensitive.

Background Art

It has been known since the pioneering work of Kohler and Milstein that it is possible to immortalize nontransformed B cells by fusing them, for example in the presence of polyethylene glycol, to an immortalizing cell line. The immortalizing cell line will typically be a "transformed" or tumor cell line. In the fused cell,

whatever it was that conferred immortality on the transformed cell is retained in the fused product.

The fusion procedure is generally conducted so that only fused cells can survive on a culture medium, thus automatically selecting only the colonies of successfully fused products. This has typically been arranged by using, as the immortalizing cells, cells of a tumor cell line that has phenotypic characteristics making it sensitive to certain medium conditions.

Unfused nontransformed cells are unable to grow, because they are not immortal. However, the medium must be chosen to take advantage of the sensitivity of the transformed cell line to the relevant conditions to prevent the growth of colonies of unfused transformed cells. A very commonly used sensitivity is the inability to grow on hypoxanthine-aminopterin-thymidine (HAT) medium, a medium which requires the presence of the enzyme hypoxanthine phosphoribosyl transferase (HPRT) in order for cells to grow. The immortalizing cell line is deficient in the production of this enzyme by virtue of its own genetic characteristics. If the fusion mixture is cultured on HAT medium, then, the immortal cells are unable to grow because they lack HPRT. Only fused cells are able to grow because they are both immortal and produce HPRT by virtue of the ability of the nontransformed cell to do so.

The fusion technique of Kohler and Milstein was an adaptation of cell fusion procedures to obtain immortalized B cells which are capable of secreting monoclonal antibodies with desired characteristics. It was employed by immunizing an animal with the antigen to

which antibody is desired, harvesting the B cells from the spleen or peripheral blood lymphocytes (PBLs), fusing the B cells with an immortalizing cell line, selecting for fused cells, and screening individual colonies of fused cells for secretion of the desired antibody.

In this classical procedure, only a small percentage of the fused cells will, in fact, secrete the antibody of the desired characteristics for two reasons: first, not all of the B cells were capable of secreting the desired antibody in the first place; and second, even if they were, the antibody-encoding genes often do not enter the genome of the fused cell in a stable manner so as to be transmitted to the progeny of original fused cell. Of course, it would not be necessary for the relevant immunoglobulin loci to be transmitted to the progeny in order for the cells to survive, since the HPRT is encoded on the X-chromosome background in all of the B cells.

The present invention offers a method to minimize the second reason for obtaining numbers of successful colonies which nevertheless do not contain genes for the desired antibody or other genomic component.

Disclosure of the Invention

The invention provides a modification of the Kohler/Milstein fusion technique which results in a higher percentage of successful fusions containing a desired genomic component. This is especially useful when the genomic component is the set of loci required to produce immunoglobulin. The DNA on which the desired genomic component resides is itself modified to contain a marker

which is capable of overcoming the sensitivity of the immortalizing cell intended to be used as a fusion partner. In this way, "background" markers, such as HPRT, native to the nontransformed cell containing the desired genomic component, are not required to permit growth on medium conditions to which the immortalizing cell line is sensitive. In an ideal case, the marker may not even be present in the background genomic complement of the nontransformed cell.

Thus, in one aspect, the invention is directed to a method to improve the percentage of colonies of fused cells that contain a desired genomic component, which fused cells are obtained by the fusion of nontransformed animal, especially mammalian or avian, cells containing said desired genomic component with cells of a transformed cell line sensitive to predetermined medium conditions which method comprises: providing said desired genomic component with a marker that overcomes the sensitivity of the cells of the transformed cell line to the medium conditions; mixing said nontransformed animal cells with cells of said transformed cell line under conditions that promote fusion, to obtain a fusion mixture; selecting for fused cell colonies by culturing said fusion mixture under said predetermined medium conditions; and screening successful colonies for the presence of said desired genomic component.

In other aspects, the invention is directed to immortalized cell lines produced by the method of the invention and to methods of producing desired products by culturing these cells.

Brief Description of the Drawings

Figures 1 A-E include photographs of the results of Southern blot analysis to characterize yHPRT and yeast genomic DNA integrated in ES clones as described in Example 2. A is the human repetitive Alu sequence. B and C are pBR-specific sequences for right and left YAC arms, respectively. D is the yeast Ty repetitive sequence. E is yeast single-copy gene LYS2.

Figures 2 A-D are photomicrographs of the results of *in situ* hybridization to detect integration of yHPRT and yeast genomic sequences in ES cell chromosomes as described in Example 2. A and B are metaphase spreads from ESY8-7 cells hybridized to biotinylated human genomic sequences. C and D represent metaphase spreads or interphase nuclei from ESY8-6 cells hybridized to biotinylated yeast repeated DNA sequences.

Figures 3 A-C demonstrate the stable retention of yHPRT during *in vitro* ES cell differentiation and transmission through the mouse germline described in Example 2.

Figures 4 A and B are photographs of an electrophoresis gel showing the expression of the human HPRT gene in various mouse tissues as described in Example 2.

Figure 5 is a diagram of a mouse breeding scheme as described in Example 3.

Modes of Carrying-Out the Invention

In general the invention method is similar to that commonly used to obtain immortalized cells capable of secreting antibodies except that the genomic component,

e.g., the loci encoding the heavy and light chains of an antibody are proximal on a DNA sequence to the gene encoding a marker such as HPRT or neomycin gene. This apparently innocent modification results in greatly enhanced percentages of colonies of immortalized fused cells that produce the product of the genomic component of choice.

As used herein, "nontransformed" cells refers to cells which are incapable of indefinite growth in culture, and are thus not immortal. These cells are not "cell lines" -- i.e., they cannot be repeatedly passaged. Examples of nontransformed cells include most normal animal, preferably mammalian or avian, cells that have already been differentiated and that are not malignant. Most commonly used are B cells, but other nontransformed cells, to the extent that they may be desirable producers of a desired protein could also be used. Thus, suitable nontransformed cells might also include T-lymphocytes, muscle cells, pituitary cells, pancreatic cells, and the like.

As used herein, "transformed" cells refer to cells that have acquired the ability to passage indefinitely and can thus be established as cell lines. A large number of cell lines is known in the art, including HeLa cells, CHO cells, COS cells and a large number of murine myeloma cell lines. In order to be useful in the method of the invention, the transformed cells must also have a characteristic which confers sensitivity to particular growing conditions, which sensitivity can be reversed by the production of one or more gene products obtainable from a nontransformed fusion partner.

"Fused cell line" refers to the products of successful fusions -- i.e., fusions wherein an immortalized or transformed cell has successfully fused with a nontransformed cell that contains a desired genomic compound. The most commonly used technique to effect cell fusion is treating with polyethylene glycol. However, other techniques are also available in the art including, for example, electroporation and fusogenic virus treatment.

A "desired genomic component" refers to a portion of DNA in the genomic complement of the nontransformed cell which is desired to be transferred to the progeny of the fused cell line. The genomic component may be an expression system for a single gene, such as, for example, the gene encoding a growth factor or hormone. In an important embodiment of the present invention the "genomic component" involves more than one expression system for more than one gene. In a particularly important application of the present technique, the relevant genomic component consists of an expression system for an immunoglobulin heavy chain and an expression system for an immunoglobulin light chain (or the appropriate portions thereof) wherein the resulting antibody is immunoreactive with a particular target antigen. It should be clear that "expression system" does not necessarily refer to cloned protein-encoding DNA manipulated so as to be operably linked to heterologous promoters, although such constructs are included. Also within the scope of "expression systems" are, for example, sections of genomic DNA which include both expression control sequences and protein encoding sequences. In

particular, expression systems for immunoglobulins envisioned in the present invention include both rearranged coding sequences for particular immunoglobulins and unrearranged genomic loci.

It is important to understand, in order to appreciate the advantage of the methods of the present invention, that colonies that result from successful fusion of the nontransformed and transformed cells do not necessarily carry the complete genomic content of the partners into the progeny. Thus it is perfectly possible for a successful fusion of, for example, a B cell with an immortalized cell to result in a successful fusion that nevertheless does not carry one or both of the immunoglobulin loci.

In the parent application herein, methods were described to obtain transgenic mice capable of forming human antibodies in response to administration of an antigen. The mice were obtained by inserting the unrearranged human heavy- and light-chain immunoglobulin loci into murine embryonic stem cells (ES cells) using vectors or yeast spheroplasts, wherein the human loci were contained on yeast artificial chromosomes (YACs) and marked with a selection marker. Both the neomycin resistance gene (the "neo" gene) and the HPRT gene are described as markers. The resulting ES cells are selected for uptake of the appropriate YAC by means of the marker used. Through use of the YAC-containing ES cells to obtain chimeric mice and subsequent cross-breeding, mice were obtained which contained unrearranged human heavy-chain and light-chain loci in their genomic complement. In these mice, the markers are contained proximal to the

immunoglobulin loci. After immunization of the transgenic mice with the desired antigen, B cells are harvested and fused with immortalizing cells to obtain colonies producing the desired antibodies. In this process, the desired genomic component (i.e., the immunoglobulin heavy- and light-chain loci) already contains markers proximal to the relevant component on the same DNA sequence.

When the foregoing fusions are conducted, if the standard method using HAT as a selection medium is used, the efficiency of obtaining successful fusion colonies which contain the desired genomic component is improved if HPRT had been used as the marker or is otherwise associated with the immunoglobulin genes. This results because within the total number of colonies obtained, there will be included, among that number, progeny that contain only the desired genomic component but not the X-chromosome of the B cell containing the "background" HPRT gene. On the other hand, if a different marker, for example neo, had been used to select for the transformation of the ES cells, but the standard HAT medium is used as a selection for the fusions of B cells with transformed cells, the successful colonies will include only fusions which contain in their progeny the background HPRT on the X-chromosome. Thus, those fusions which do not contain one or both immunoglobulin loci, but only the background HPRT gene, will not be weeded out of the pool; and the pool will include fusions with the X-linked HPRT, but neither or only one of the Ig loci.

Alternatively, since the transforming cells are also sensitive to the neomycin antibiotic (or G418-containing media), media containing G418 could be used as a

selection medium for the B cell/myeloma fusion provided the neo gene was used as a marker or otherwise included on the inserted genome for the ES cell modification.

It should be evident that the provision of a marker proximal to immunoglobulin loci need not be an incidental result of the use of markers in transforming ES cells in selecting for successful transformants. The marker can be provided, for example, adjacent a desired locus either in addition to another marker with respect to ES cell transformation or can simply be included when the locus is inserted into fertilized eggs by microinjection. In this procedure, no selection is needed; the offspring are simply tested as appropriate to see whether the desired characteristics have been successfully included in the genome. Nevertheless, the loci representing the desired characteristics can readily be provided a desired marker using standard recombinant techniques.

The method can further be refined by using multiple markers when multiple genes comprise the genomic component desired to be transmitted to the fused cell progeny. For example, in connection with the transfer of immunoglobulin genes, the light chain might be provided with a neo marker and the heavy chain provided with an HPRT marker. In the fusion, a G418-containing HAT medium would be used for selection and surviving progeny must contain at least the immunoglobulin light chain and either the immunoglobulin heavy chain or the X-chromosome-based HPRT marker.

Alternatively, or in addition, the gene for hygromycin resistance can be used as a marker in the method of the invention.

A more straightforward illustration of the method of the invention can employ nontransformed cells that have sufficient survival characteristics that they can be selected for successful homologous recombination. B cells are not preferred for use in this illustration since their survival time in culture appears to be unacceptably short. However, some T cell clones and other cells which exhibit useful properties or produce useful products and which are not immortal are able to survive sufficient passages in culture that they are useful candidates for homologous recombination to insert a marker. The nontransformed primary cells are treated under conditions that effect DNA uptake with a vector which contains, for example, the neo-resistance gene bracketed by sequences homologous to those found immediately upstream or downstream of the genomic component disired. Successful transformants which incorporate the neo gene into the DNA sequence that includes the genomic component by homologous recombination can then be selected by growth on G418-containing medium. The resulting nontransformed primary cells, then contain G418 resistance encoded proximal to the regions that contain the desired genomic compenent.

In the standard fusion protocol, the immortalizing cell line, sensitive to G418, requires successful fusion to survive on G418-containing medium. Successfully fused cells capable of surviving on medium containing G418 must acquire this capability from the nontransformed cell. Since the neo-resistance gene travels with the desired genomic component, it is assured that this component is transferred to the progeny.

In a more typical example, where more traditional selection medium is used for the fused product, HPRT is introduced proximal to the desired genomic component. In this case, direct selection after homologous recombination in many primary cells cannot conveniently be used since such cells in general already contain an HPRT gene on the X chromosome. However, as described above, transgenic animals have been prepared by inserting immunoglobulin genes into fertilized eggs or ES cells. In this manner, HPRT can be introduced directly along with the desired genes. In this embodiment, both heavy and light chain encoding genes can be assured to be proximal to the HPRT encoding region.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Cloning of Human Heavy Chain Locus using Yeast Artificial Chromosomes

A. Production of Yeast Artificial Chromosome (YAC) containing human heavy chain

An SpeI fragment, spanning the human heavy chain VH6-D-J-Cμ-Cδ region (Berman et al. (1988), EMBO J. 7: 727-738; see Figure 4) is isolated from a human YAC library (Burke, et al., Science, 236: 806-812) using DNA probes described by Berman et al. (1988) EMBO J. 7:727-738. One clone is obtained which is estimated to be about 100 kb. The isolated YAC clone is characterized by pulsed-field gel electrophoresis (Burke et al., supra; Brownstein et al., Science, 244: 1348-1351), using

radiolabelled probes for the human heavy chain (Berman et al., supra).

B. Introduction of YAC clones into embryos or ES Cells

High molecular weight DNA is prepared in agarose plugs from yeast cells containing the YAC of interest (i.e., a YAC containing the aforementioned SpeI fragment from the IgH locus). The DNA is size-fractionated on a CHEF gel apparatus and the YAC band is cut out of the low melting point agarose gel. The gel fragment is equilibrated with polyamines and then melted and treated with agarase to digest the agarose. The polyamine-coated DNA is then injected into the male pronucleus of fertilized mouse embryos which are then surgically introduced into the uterus of a psueudopregnant female as described above. The transgenic nature of the newborns is analyzed by a slot-blot of DNA isolated from tails and the production of human heavy chain is analyzed by obtaining a small amount of serum and testing it for the presence of Ig chains with rabbit anti-human antibodies.

As an alternative to microinjection, YAC DNA is transferred into murine ES cells by ES cell: yeast protoplast fusion (Traver et al., (1989) Proc. Natl. Acad. Sci., USA, 86:5898-5902; Pachnis et al., (1990), ibid 87: 5109-5113). First, the neomycin-resistance gene from pMC1Neo or HPRT or other mammalian selectable marker and a yeast selectable marker are inserted into nonessential YAC vector sequences in a plasmid. This construct is used to transform a yeast strain containing the IgH YAC, and pMC1Neo (or other selectable marker) is integrated into vector sequences of the IgH YAC by homologous

recombination. The modified YAC is then transferred into an ES cell by protoplast fusion (Traver et al. (1989); Pachnis et al., 1990), and resulting G418-resistant ES cells (or exhibiting another selectable phenotype) which contain the intact human IgH sequences are used to generate chimeric mice. Alternatively, a purified YAC is transfected, for example by lipofection or calcium phosphate-mediated DNA transfer, into ES cells.

Example 2

Introduction of Human Ig Genes into Mice

A. Cloning of Human Ig Genes in Yeast

1. Identification and characterization of a human IgH YAC clone containing VH, D, JH, mu and delta sequences:

PCR primers for the human VH6 gene (V6A= 5' GCA GAG CCT GCT GAA TTC TGG CTG 3' and V6B= 5' GTA ATA CAC AGC CGT GTC CTG G 3') were used to screen DNA pools from the Washington University human YAC library (Washington University, St. Louis, MO). Positive pools were subsequently screened by colony hybridization and one positive microtiter plate well, A287-C10, was identified. Two different sized (205 kb and 215 kb) VH6-containing YACs were isolated from the microtiter well.. In addition to VH6, the smaller of the two IgH YACs , A287-C10 (205 kb), hybridized to probes for the following sequences: delta, mu, JH, D, VH1, VH2, and VH4. The larger of the two IgH YACs, A287-C10 (215 kb), hybridized to the following probes: delta, JH, D, VH1, VH2, and VH4, but not to mu. The YACs contained sequences from at least 5 VH genes including two VH1 genes, one VH2, one VH4 and one

VH6 gene. Analysis of restriction digests indicated that the 205 kb YAC contains a deletion (about 20 kb size) that removes some, but not all of the D gene cluster, with the remainder of the YAC appearing to be intact and in germline configuration. PCR and detailed restriction digest analysis of the 205 kb YAC demonstrated the presence of several different D gene family members. The 215 kb YAC appeared to contain the complete major D gene cluster but had a deletion (about 10 kb) that removed the mu gene. This deletion does not appear to affect the JH cluster or the enhancer located between JH and mu genes.

The putative progenitor of the above two related IgH YACs, a YAC of about 225-230 kb containing the entire genomic region between the VH2 gene and the delta gene (Shin et al., 1991, supra) (see Figure 4), had not been identified in the A287-C10 microtiter well. Hence, an earlier aliquot of the A287-C10 microtiter plate well was examined in order to search for the progenitor YAC under the assumption that it was lost during passaging of the library. The A287-C10 microtiter well was streaked out (Washington University, St. Louis, MO), and 2 of 10 clones analyzed contained a 230 kb IgH YAC with another apparently unrelated YAC. Clone 1 contained in addition the IgH YAC, an approximately 220 kb YAC and clone 3 in addition contained an approximately 400 kb YAC. The IgH YAC contained mu, the complete D profile (based on a BamHI digest, see below) and JH. The IgH YAC from clone 1 was physically separated from the unrelated YAC by meiotic segregation in a cross between A287-C10/AB1380 and YPH857 (genotype = MATα ade2 lys2 ura3 trp1 HIS5 CAN1 his3 leu2

cyh2, to yield A287-C10 (230 kb)/MP 313 (host genotype = MATα ade2 leu2 lys2 his3 ura3 trp1 can1 cyh2).

2. Targeting of the A287-C10 kb YAC with a mammalian selectable marker, HPRT:

A YAC right arm targeting vector called pLUTO (15.6 kb) was generated by subcloning a human HPRT minigene contained on a 6.1 kb BamHI fragment (Reid et al., Proc. Natl. Acad. Sci. USA 87:4299-4303 (1990)) into the BamHI site in the polylinker of pLUS (Hermanson et al., Nucleic Acids Research 19:4943-4938 (1991)). A culture of A287-C10/AB1380 containing both the 230 kb IgH YAC and an unrelated YAC was transformed with linearized pLUTO and Lys+ transformants were selected. The Lys+ clones were screened by colony hybridization for the presence of mu. One clone was identified which contained a single YAC of approximately 245 kb which hybridized to probes for mu, HPRT and LYS2.

Southern analysis of the 230 kb A287-C10 YAC targeted with pLUTO was carried out using a variety of probes to demonstrate the intact, unrearranged nature of the cloned, human IgH sequences. In most cases, the results of BamHI, HindIII and EcoRI digests were compared to restriction data for WI38 (a human embryonic fetal lung-derived cell line), the 205 kb and 215 kb deletion-derivatives of A287-C10 and to published values. The diversity (D) gene profile determined by hybridization with a D region probe (0.45 NcoI/PstI fragment; Berman et al., 1988) demonstrated the expected four D gene segments (D1-D4 (Siebenlist et al., 1981; Nature 294:631-635). For example, with BamHI, four restriction fragments, 3.8 kb,

4.5 kb, 6.9 kb and 7.8 kb, were observed in A287-C10 and WI38. WI38 had one additional larger band, presumed to originate from the chromosome 16 D5 region (Matsuda et al., 1988, EMBO 7:1047-1051). PCR and Southern analysis with D family-specific primers and probes demonstrated in the 215 kb deletion-derivative YAC (which appeared to have an intact D region with the same restriction pattern as the 230 kb YAC) the presence of 2 to 4 members of each of the following D gene families: DM, DN, DK, DA, DXP and DLR. The J-mu intronic enhancer, which was sequenced from cloned PCR products from the A287-C10 230 kb YAC (primers EnA = 5' TTC CGG CCC CGA TGC GGG ACT GC 3' and EnB1 = 5' CCT CTC CCT AAG ACT 3') and determined to be intact, also generated single restriction fragments of approximately the predicted sizes with BamHI, EcoRI and HindIII when probed with the 480 bp PCR product. The JH region was evaluated with an approximately 6 kb BamHI/HindIII fragment probe spanning DHQ52 and the entire JH region (Ravetch et al., 1981, Cell 27:583-591). A287-C10 generated restriction fragments of approximately the expected sizes. Furthermore, the same-sized restriction fragments were detected with the enhancer and the JH probes (Ravetch et al., supra; Shin et al., 1991, supra). The approximately 18 kb BamHI JH fragment detected in A287-C10 and WI38 also hybridized to a 0.9 kb mu probe sequence (Ravetch et al., supra). Hybridization with the 0.9 kb EcoRI fragment mu probe (Ravetch et al., supra) showed restriction fragments of approximately the expected sizes (Ravetch et al., supra; Shin et al., supra: > 12 kb BamHI (approximately 17 kb expected); 0.9 kb EcoRI (0.9 kb expected) and approximately 12 kb HindIII (approximately

11 kb expected). WI38 gave the same-sized BamHI fragment as A287-C10. The JH and DHQ52 regions were sequenced from both of the deletion derivative YACs and both were in germline configuration. Delta was analyzed with an exon 1 PCR product (containing the approximately 160 bp region between primers D1B= 5' CAA AGG ATA ACA GCC CTG 3' and D1D = 5' AGC TGG CTG CTT GTC ATG 3'); restriction fragments for A287-C10 were close to those expected from the literature (Shin et al., supra) and to those determined for WI38. The 3' cloning site of the YAC may be the first EcoRI site 3' of delta (Shin et al., supra) or another EcoRI site further 3'. VH gene probes for VH1, VH4 and VH6 (Berman et al., supra), and for VH2 (Takahashi et al., 1984, Proc. Nat. Acad. Sci. USA 81:5194-5198) were used to evaluate the variable gene content of the YAC. A287-C10 contains two VH1 genes that approximate the predicted sizes (Shin et al., supra; Matsuda et al., 1993, supra); restriction analysis with the three enzymes gave close to the expected fragment sies; e.g. with EcoRI observed bands are 3.4 and 7.8 kb (expected are 3.4 and 7.2 kb). The predicted size EcoRI fragments for VH4 (5.3 kb observed, 5.1 kb expected) and for VH6 (0.8 kb observed, 0.9 kb expected) (Shin et al., supra; Matsuda et al., supra) were present in A287-C10. The expected size EcoRI fragment was seen for VH2 (5.5 kb observed, 5.4 kb expected), but the BamHI and HindIII fragments were different from those predicted. Coincident hybridization of the BamHI and HindIII fragments with a pBR322 probe suggested that the EcoRI site which is at the 5' end of the VH2 gene (Shin et al., supra) is the 5' cloning site, thus eliminating the natural 5' HindIII site and BamHI sites. The overall size

of the YAC insert (estimated to be approximately 220 kb) fits well with the predicted size for an intact, unrearranged segment starting at the 5' end of the 3'-most VH2 gene and extending to an EcoRI site 3' of the delta locus (Shin et al., supra).

3. Identification and characterization of IgK YACs containing CK and VK sequences:

Two YACs were identified in a screen of pulsed-field gel (PFG) pools from the Washington University (St. Louis, MO) human YAC library with a probe from the human kappa constant region (CK) gene (2.5 kb EcoRI fragment ATCC No. 59173, Parklawn Dr., Rockville, MD). The YACs, designated A80-C7 (170 kb) and A276-F2 (320 kb), contain the kappa deleting element kde, CK, JK and the C-J intronic enhancer and extend 3' beyond kde. Extending 5' from JK, the YACs also contain the B1, B2 and B3 VK genes determined by hybridization and/or PCR, and possibly other VK sequences. The A80-C7/AB1380 strain housed, in addition to the IgK YAC, an unrelated YAC of similar size. Therefore, meiotic segregation was used to separate these YACs; A80-C7 was crossed to YPH857 and a meiotic product was obtained which contained only the IgK YAC (MP8-2; host genotype = α ade2 leu2 his3 his5 lys2 ura3 trp1 can1 cyh2). The A80-C7 and A276-F2 YACs have been targeted with pLUTO to incorporate the human HPRT minigene into the YAC right vector arm.

Restriction analysis of the IgK YACs A80-C7 and A276-F2 using a number of enzymes supports the conclusion that both YACs are unrearranged (i.e., in germline configuration). For example, BamHI digestion followed by

hybridization with the CK probe demonstrates the expected 13 kb restriction fragment (Klobeck et al., Biol. Chem. Hoppe-Seyler 370:1007-1012 (1989)). The same-sized band hybridizes to a JK probe (a 1.2 kb PCR product using primer set to amplify the JK1-5 region), as predicted from the genomic map (Klobeck et al., supra). The B3 class IV gene (probe is a 123 bp PCR product from the B3 gene) gives a 4.9 kb BamHI and a 2.2 kb BglII fragment, close to the published values of 4.6 kb and 2.3 kb, respectively (Lorenz et al., Molec. Immunol. 25:479-484 (1988)). PCR analysis of both IgK YACs as well as human genomic DNA for the following kappa locus sequences revealed the predicted band sizes: Kde (120 bp), CK (304 bp), C-J intronic enhancer (455 bp), JK1-5 (1204 bp), B3 VK (123 bp) and B1 VK pseudogene (214 bp). Sequences used to design PCR primers for the CK, JK and C-J enhancer regions are from Whitehurst et al., Nucl. Acids. Res. 20:4929-4930 (1992); Kde is from Klobeck and Zachau, Nucl. Acids. Res. 14:4591-4603 (1986); B3 is from Klobeck et al., Nucl. Acids. Res. 13:6515-6529 (1985); and B1 is from Lorenz et al., supra.

B. Introduction of 680 kb yHPRT YAC into ES Cells

1. Culture of yHPRT yeast strain and preparation of yeast spheroplasts

The 680 kb yHPRT is a YAC containing a functional copy of the human hypoxanthine phosphoribosyltransferase (HPRT) gene cloned from a YAC library, as described in Huxley, et al. (1991) Genomics 9:742-750. The yeast strain containing the yHPRT was grown in uracil and tryptophan deficient liquid media, as described in Huxley, et al. (1991) supra.

To prepare the yeast spheroplasts, a 400 ml culture of yeast containing yHPRT was spun down and the yeast pellet was washed once with water and once with 1 M sorbitol. The yeast pellet was resuspended in SPEM (1 M sorbitol, 10 mM sodium phosphate pH 7.5, 10 mM EDTA pH 8.0, 30 mM β-mercaptoethanol) at a concentration of 5 x 10^8 yeast cells/ml. Zymolase 20T was added at a concentration of 150 μg/ml of yeast cells, and the culture was incubated at 30°C until 90% of the cells were spheroplasts (usually for 15-20 minutes). The cells were washed twice in STC (1 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) and resuspended in STC at a concentration of 2.5 x 10^8/ml.

2. Culture of E14TG2a ES Cells

HPRT-negative ES cell line E14TG2a was cultured on mitomycin C-treated embryonic fibroblast feeder layers as described by Koller, *et al.* PNAS (1989) 86:8932-8935.

3. Fusion of ES Cells and Yeast Spheroblasts

Exponentially growing E14TG2a ES cells growing on gelatin-coated dishes were trypsinized and washed three times with serum-free DMEM. A pellet of 2.5x10^8 yeast spheroplasts was carefully overlaid with 5x10^6 ES cells which were spun down onto the yeast pellet. The combined pellet was resuspended in 0.5 ml of either 50% polyethylene glycol (PEG) 1500 or 50% PEG 4000 (Boeringer Mannheim) containing 10 mM CaCl2. After 1.5 minutes incubation at room temperature or at 37°C, 5 ml of serum-free DMEM were added slowly, and the cells were left at room temperature for 30 minutes. The cells were then

pelleted and resuspended in 10 ml of ES cell complete medium (as previously described) and were plated onto one 100 mm plate coated with feeder cells. After 24 hours the medium was replaced with fresh medium. Forty-eight hours post-fusion, HAT (ES media containing $1x10^{-4}$ M hypoxanthine, $4x10^{-7}$ M aminopterin, $1.6x10^{-5}$ thymidine) selection was imposed. HAT-resistant ES colonies were observed 7-10 days post-fusion in the plates from both the different fusion conditions used. yHPRT-ES ("ESY") fusion colonies were picked and plated onto feeder-coated wells, and expanded for further analysis.

4. Analysis of YAC DNA Integrated into yHPRT-ES Fusion Clones

DNA extracted form 23 yHPRT-ES fusion colonies was digested with HindIII and subjected to Southern blot analysis (Figure 1) using the probes: a human repetitive Alu sequence (A); pBR322-specific sequences for the right (B) and left (C) YAC vector arms; yeast Ty repetitive sequence (D); yeast single copy gene LYS2 (E). The human HPRT probe, a 1.6 kb full length cDNA (Jolly et al., Proc. Natl. Acad. Sci. USA 80:477-481 (1983)) was used to confirm the presence of the human HPRT gene in ESY clones. The Alu probe was a 300 bp BamHI fragment from the BLUR8 Alu element in pBP63A (Pavan et al., Proc. Natl. Acad. Sci. USA 78:1300-1304 (1990)). The right and left vector arm probes were pBR322-derived BamHI-PvuII 1.7 and 2.7 kb fragments, respectively, which correspond to the vector sequences in pYAC4 (scheme a, b (Burke et al., in: Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Guthrie and Fink, eds., Academic Press,

194:251-270 (1991)). The 4.5 kb fragment, detected by the right arm probe, spans the region between the HindIII site at the telomere 5' end and the first HindIII site within the human insert (scheme a). The 3 kb and 4.1 kb fragments detected by the left end probe correspond to the region between the HindIII site at the telomere end and the HindIII site 5' of the yeast sequences, and the region spanning from the HindIII site 3' of the centromere into the human insert, respectively (scheme b). The difference in the hybridization intensity of these two bands relates to the difference in the amount of homology between these fragments and the probe. The yeast Ty repetitive probe (Philippsen et al., in Gene Expression in Yeast, Proceedings of the Alko Yeast Symposium, Helsinki, Korhola and Vaisanen, eds., Foundation for Biotechnical and Industrial Fermentation Research, 1:189-200 (1983)) was a 5.6 kb XhoI fragment isolated from Ty1-containing pJEF742 which could also detect the 3' HindIII fragment of Ty2, due to the homology between the two elements. The LYS2 gene probe was a 1.7 BamHI fragment from pLUS (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)).

Hybridization with a human HPRT probe (full length 1.6 kb cDNA probe) demonstrated that all the clones analyzed contained the same 15, 7 and 5 kb exon-containing fragments of the human HPRT gene as the yHPRT YAC. Reprobing the same blots with a human repetitive Alu sequence 300 bp probe indicated that all the clones analyzed contained most, if not all, the Alu-containing fragments present in yHPRT (Figure 1A). These data indicate that in most of the clones analyzed the 680 kb human insert had not been detectably rearranged or deleted

upon integration into the ES cell genome. Integration of YAC vector sequences was examined using probes specific for the vector arms. Rehybridization of the same blots with a probe for the right YAC vector arm, detecting a 4.5 kb HindIII fragment, indicated that in 10 out of 23 of the clones analyzed, the right YAC arm up to the telomere was still intact and unrearranged and linked to the human insert (Figure 1B) thus providing further evidence for the integrity of the YAC in these clones. The left arm probe detected the 3 kb and 4.1 kb HindIII yHPRT fragments in 18 out of the 20 clones analyzed (Figure 1C), indicating a high frequency of left arm retention.

The structural integrity of yHPRT in ESY clones was further evaluated for two clones (ESY 5-2 and 8-7) using pulsed-field gel restriction analysis. In yeast carrying yHPRT, five Sfi fragments of the following approximate sizes were defined by different probes: 315 kb (Alu, left arm), 145 kb (Alu, HPRT); 95 kb (Alu, right arm), 70 and 50 kb (Alu only). In both ES clones, the internal HPRT and Alu-specific fragments were similar in size to the yHPRT fragments. The end fragments detected for both clones were larger than those in yHPRT, as expected for YACs integrated within a mouse chromosome: 185 and 200 kb for the right end fragment, respectively, and over 800 kb for the left end fragment for both clones. These data, together with the Alu profile, provide additional evidence for the retention of the structural integrity of the YAC in these clones. These studies were complemented by fluorescence in-situ hybridization carried out on ESY 8-7 (Figure 2 A, B) and ESY 8-6 metaphase chromosome spreads in which a single integration site was

detected for the human sequences. Photomicrographs of representative metaphase spreads (Figure 2 A, B, C) or interphase nuclei (Figure 2D) from ESY 8-7 cells (Figure 2 A, B) hybridized with biotinylated human genomic sequences and ESY 8-6 cells (Figure 2 C, D) hybridized with biotinylated yeast repeated DNA sequences. The human probe was generated from human genomic placental DNA (Clontech, Palo Alto, CA). The yeast probe consisted of a mix of DNA fragments encoding the yeast repeated elements; delta (a 1.08 kb Sau3A fragment of pdelta6 (Gafner et al., EMBO J. 2:583-591 (1983)) and Ty (a 1.35 kb EcoRI-SaII fragment of p29 (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)), the rDNAs (a 4.6 kb BgIIIk-A L90 and a 4.4 kb BgIII-B L92 fragment (Keil and Roeder, Cell 39:377-386 (1984)), and the Y' telomere elements (2.0 and 1.5 kb BgIII-HindIII fragments of p198 (Chan and Tye, Cell 33:563-573 (1983)). Hybridization of sequences on chromosome metaphase spreads with biotinylated probes and detection by Avidin-FITC followed by biotin-anti-Avidin and Avidin-FITC amplification was carried as described by Trask and Pinkel, Methods Cell Biol. 30:383-400 (1990), using a Zeiss Axiophot microscope. Chromosomes were counterstained with propidium iodide. The photomicrographs shown are representative of 95% of the metaphase spreads or interphase nuclei scanned in three independent experiments carried out with the human or the yeast probes. A single integration site was detected for the human sequences.

The same blots were also probed with the yeast Ty repetitive element sequence to detect the presence of yeast genomic DNA sequences in the ESY clones (Figure 1

D). Whereas some of the clones were found to contain most of the Ty-containing fragments present in the parental yeast strain, some of the clones were found to have a very small fraction, if at all, of the Ty-containing fragments. These results indicate that in some ES clones, although the YAC DNA is integrated intact, little or no yeast genomic DNA was integrated. To determine if the yeast chromosomal DNA was integrated at single or multiple sites within the ES cell genome, fluorescent in-situ hybridization was performed on ESY clone 8-6 which had a complete Ty profile. A single integration site was detected using a combined yeast repetitive probe (Figure 2 C, D), indicating that within the limits of resolution, all yeast DNA fragments integrated in one block.

Using the ability of ES cells to undergo in vitro orderly differentiation, YAC stability and the effect of integrated DNA on the pluripotency of ES cells was investigated. Four ES clones, containing different amounts of yeast DNA (ESY 5-2, 3-6, 8-6 and 8-7) exhibited a differentiation pattern indistinguishable from that of unfused ES cells: formation of embryoid bodies giving rise to a variety of differentiated cell types (Figure 3 A). Southern blot analysis was performed on DNA extracted from differentiated ESY 5-2, 3-6, 8-5 and 8-6 (20 μg) and yHPRT in AB1380 (40 ng) using (a) a human Alu probe; (b) yeast Ty sequences. ES clones were induced to form embryoid bodies by culturing them as aggregates in suspension for 10-14 days as described by Martin and Evans, Cell 6:467-474 (1975). Following their reattachment to tissue culture substratum, ESY-derived embryoid bodies gave rise to differentiated cell types. YAC and yeast DNA sequences

were stably retained by the differentiated ES clones during 40 days of culture in non-selective medium, demonstrating that the stably integrated foreign DNA did not impair the pluripotency of the ES cells (Figure 3 B). The differentiated cultures maintained a functional human HPRT gene as evidenced by their normal growth and differentiation when transferred to HAT-selective medium.

5. Generation of chimeric mice from yHPRT-ES cell lines

The ability of ESY cells to repopulate mice, including the germline, was demonstrated by microinjection of ES cells into mouse blastocysts and the generation of chimeric mice. ESY cells were microinjected into C57BL/6J mouse blastocysts, and chimeric mice were generated as previously described. Chimeric males were mated with C57BL/6J females and germline transmission was determined by the presence of agouti offspring. Genomic DNA prepared from the tails of the chimeric mice were analyzed for the presence of the yHPRT DNA in the mouse genome by PCR analysis. The presence of the YAC left arm was analyzed using the two priming oligonucleotides,

5' TTCTCGGAGCACTGTC CGACC

and

5' CTTGCGCCTTAAACCAACTTGGTACCG,

which were derived, respectively, from the pBR322 sequences and the SUP4 gene within the YAC left vector arm. A 259 bp PCR product was obtained from the analysis of the yeast containing yHPRT and the ESY cell lines. PCR analysis of tail DNA prepared from 18 chimeric mice generated from ESY cell lines ESY3-1 ESY3-6 and ESY5-2, gave rise to the expected PCR product, thus indicating the

presence of the YAC left vector arm in the genome of the chimeric mice.

6. Germline transmission of yHPRT

Chimeric males, with coat color chimerism of 30-60%, derived from the ESY cell lines ESY3-1 and ESY5-2 were set up for mating for germline transmission evaluation, i.e. to determine whether the genetic modification was passed via the germ cells (sperm or oocytes) to the progeny of the animals. Three of the chimeric ESY3-1 derived males, 394/95-1, 394/95-2 and 411-1 transmitted the ES cell genome to their offspring at a frequency of 20%, 30% and 30%, respectively. Southern blot analysis of tail DNA from the agouti pups indicated the presence of the yHPRT in the genome of three mice, 4-2, 4-3 and 5-1, derived from the 394/395-2 chimera. The Alu profile obtained from such analysis was indistinguishable from that of the parent ES3-1 cell line (Figure 3 C), demonstrating that the 680 kb human insert was transmitted faithfully through the mouse germline.

Using a human HPRT-specific PCR assay on mRNA-derived cDNAs from a yHPRT-containing offspring, the expression of the human HPRT gene in all the tissues tested was detected (Figure 4 A and B), thus demonstrating the transmitted YAC retained its function with fidelity. In this experiment, human HPRT mRNA was detected by reverse transcription (RT)-PCR in ES, ESY 3-1 and Hut 78 (human) cells, spleen and liver from a control mouse (C) or the 4-3 agouti offspring (derived from the 394/95-2 chimera) and a sample containing no template DNA (indicated as "-" in Figure 4A). Reverse transcription of

poly (A+) RNA and PCR amplification of specific cDNA sequences were performed using the cDNA Cycle Kit (Invitrogen). Specific amplification of a 626 bp fragment from human HPRT cDNA in the presence of murine HPRT cDNA was performed as outlined by Huxley et al, supra. Integrity of all RNA samples was demonstrated by PCR amplification of cDNAs for the mouse γ-interferon receptor. The primers used to amplify a 359 bp fragment were: GTATGTGGAGCATAACCGGAG and CAGGTTTTGTCTCTAACGTGG. The human HPRT and the γ-interferon receptor primers were designed to eliminate the possibility of obtaining PCR products from genomic DNA contamination. PCR products were analyzed by electrophoresis and visualized with ethidium bromide. The size markers are 1 kb ladder (BRL). The results of detection of mouse γ-interferon receptor mRNA by RT-PCR in the samples described above are shown in Figure 4B. The specific human HPRT mRNA was also detected in the other tissues tested (brain, kidney and heart) derived from the 4-3 mouse. Comparable steady-state levels of mouse and human HPRT mRNA were detected in the liver of yHPRT-containing progeny. These results indicate that the uptake of as much as 13 megabases of yeast genomic DNA was not detrimental to proper development, germline transmission or gene expression.

The above results demonstrate that yeast spheroplasts are an effective vehicle for the delivery of a single copy large molecular weight DNA fragment into ES cells and that such molecules are stably and functionally transmitted through the mouse germline. The Alu profiles, complemented by PFGE analysis and in situ hybridization for some of the ES clones, strongly argue that the

majority of the clones contained virtually all the human insert in unrearranged form (i.e. in "germline configuration"), with a high frequency of clones (40%) also retaining both YAC arms. The significant uptake of yeast genomic DNA was not detrimental to proper differentiation of ES cells in vitro and in vivo and did not prevent germline transmission or gene expression. Using these methods, one can transmit large fragments of genomic DNA as inserts into non-human animal genomes, where the inserts may be transmitted intact by germline transmission. Therefore, a wide variety of xenogeneic DNA can be introduced into non-human hosts such as mammals, particularly small laboratory animals, that may impart novel phenotypes or novel genotypes. For example, one can provide in small laboratory animals genes of a mammal, such as a human, to study the etiology of a disease, the response to human genes to a wide variety of agents. Alternatively, one can introduce large loci into a mammalian host to produce products of other species, for example humans, to provide human protein sequences of proteins such as immunoglobulins, T-cell receptors, major histocompatibility complex antigens, etc.

Introduction of heavy chain YAC A287-C10 and kappa chain YAC A80-C into ES cells and Embryos

Yeast containing the human heavy chain YAC A287-C10 targeted with pLUTO (yA287-C10) were spheroplasted and fused with the HPRT-deficient ES cell line E14.1TG3B1 as described above. Ten HAT-resistant ES (ESY) clones (2B, 2C, 2D, 3A, 3B, 5C, 1125A, 1125E, 100/1500 and 100/4000) were picked and were expanded for DNA analysis.

Evaluation of the integrated YAC was performed by Southern blot analysis of HindIII-digested DNA from these clones, using human heavy chain probes for the D, J_H, μ, and VH2 regions, decribed above. All ESY clones were found to contain the expected > 10 kb J_H and μ fragments. All ESY clones except 2D and 5C clones, were found to contain the 4.8 kb VH2 kb fragment. All ESY clones, except 2D and 3B were found to contain the expected 10 and 7.6 kb D gene fragments. Yeast genomic sequences were detected by hybridization to the yeast repetitive Ty element in all ESY clones except 2B, 2D, 100/1500 and 5C. ESY clones 2B, 3A and 5C were microinjected into C57B/6 blastocysts as described above and chimeric mice (10 from 2B clone, 1 from 3A clone and 1 from 5C clone) were generated. Southern blot analysis of tail DNA from 10 of these chimeric animals, indicated the presence of most, if not all, of the apparent 10 Alu fragments, detected in yA287-C10 in yeast, as well as the presence of VH_2 and D gene fragments. The generated chimeric mice were bred with C57BL16J mice for germline transmission evaluation. A chimeric male 78K-3 derived from the 2B clone transmitted the ES cell genome to its offspring at a frequency of 100%. Southern blot analysis of tail DNA from 4 out of 6 agouti mice pups indicated the presence of human heavy chain sequences.

Fusion experiments with yeast containing the human kappa chain YAC A80-C7 targeted with pLUTO (yA80-C7) with E14.1TG3B1 ES cells generated 2 HAT-resistant ESY clones: M4.4.1 and M5.2.1. Southern blot analysis of HindIII-digested DNAs from these clones revealed the presence of all the apparent 10 Alu fragments detected in

yA80-C7 in yeast. In both clones yeast genomic sequences were integrated. ESY clones were microinjected into C57B1/6J blastocysts and chimeric mice were generated.

Example 3

Crossbreeding of transgenic mice

A. Generation of human monoclonal antibody producing mice

Mice containing the human immunoglobulin locus are mated to mice with inactivated murine immunoglobulin genes to generate mice that produce only human antibodies. Starting with four heterozygous strains, three generations of breeding are required to create a mouse that is homozygous for inactive murine kappa and heavy chain immunoglobulins, and heterozygous for human heavy and kappa chain immunoglobulin loci. The breeding scheme is shown in Figure 5.

Example 4

Production of Human Monoclonal Antibodies

A. Immunization of mice

Germline chimeric mice containing integrated human DNA from the immunoglobulin loci are immunized by injection of an antigen in adjuvant. The mice are boosted with antigen 14 days after the primary immunization, repeated after 35 and 56 days. A bleed is done on the immunized animals to test the titer of serum antibodies against the immunizing antigen. The mouse with the highest titer is sacrificed, and the spleen removed.

B. Fusion of splenocytes

Myeloma cells used as the fusion partner for the spleen cells are thawed 6 days prior to the fusion, and grown in tissue culture. One day before the fusion, the cells are split into fresh medium containing 10% fetal calf serum at a concentration of 5 x 10^5 cells/ml. On the morning of the fusion the cells are diluted with an equal volume of medium supplemented with 20% fetal calf serum and 2X OPI (3 mg/ml oxaloacetate, 0.1 mg/ml sodium pyruvate and 0.4 IU/ml insulin) solution.

After sacrificing the mouse, the spleen is aseptically removed, and placed in a dish with culture medium. The cells are teased apart until the spleen is torn into fine pieces and most cells have been removed. The cells are washed in fresh sterile medium, and the clumps allowed to settle out.

The splenocytes are further washed twice by centrifugation in medium without serum. During the second wash, the myeloma cells are also washed in a separate tube. After the final wash the two cell pellets are combined, and centrifuged once together.

A solution of 50% polyethylene glycol (PEG) is slowly added to the cell pellet while the cells are resuspended, for a total of two minutes. 10 ml of prewarmed medium is added to the cell solution, stirring slowly for 3 minutes. The cells are centrifuged and the supernatant removed. The cells are resuspended in 10 ml of medium supplemented with 20% fetal calf serum, 1X OPI solution and 1X AH solution (58 μM azaserine, 0.1 mM hypoxanthine). The fused cells are aliquoted into 96-well plates, and cultured at 37° for one week.

Supernatant is aseptically taken from each well, and put into pools. These pools are tested for reactivity against the immunizing antigen. Positive pools are further tested for individual wells. When a positive well has been identified, the cells are transferred from the 96-well plate to 0.5 ml of medium supplemented with 20% fetal calf serum, 1X OPI, and 1X AH in a 24-well plate. When that culture becomes dense, the cells are expanded into 5 ml, and then into 10 ml. At this stage the cells are sub-cloned so that a single antibody producing cell is in the culture.

In accordance with the above procedures, a chimeric non-human host, particularly a murine host, may be produced which can be immunized to produce human antibodies or analogs specific for an immunogen. In this manner, the problems associated with obtaining human monoclonal antibodies are avoided, because the transgenic host can be immunized with immunogens which could not be used with a human host. Furthermore, one can provide for booster injections and adjuvants which would not be permitted with a human host. The resulting B-cells may then be used for immortalization for the continuous production of the desired antibody. The immortalized cells may be used for isolation of the genes encoding the immunoglobulin or analog and be subjected to further molecular modification by methods such as in-vitro mutagenesis or other techniques to modify the properties of the antibodies. These modified genes may then be returned to the immortalized cells by transfection to provide for a continuous mammalian cellular source of the desired antibodies. The subject invention provides for a

convenient source of human antibodies, where the human antibodies are produced in analogous manner to the production of antibodies in a human host. The animal host cells conveniently provide for the activation and rearrangement of human DNA in the host cells for production of human antibodies.

In accordance with the subject invention, human antibodies can be produced to human immunogens, eg. proteins, by immunization of the subject host mammal with human immunogens. The resulting antisera will be specific for the human immunogen and may be harvested from the serum of the host. The immunized host B cells may be used for immortalization, eg. myeloma cell fusion, transfection, etc. to provide immortal cells, eg. hybridomas, to produce monoclonal antibodies. The antibodies, antiserum and monoclonal antibodies will be glycosylated in accordance with the species of the cell producing the antibodies. Rare variable regions of the Ig locus may be recruited in producing the antibodies, so that antibodies having rare variable regions may be obtained.

Claims

1. A method to increase the percentage of colonies of fused cells that contain a desired genomic component, which fused cells are obtained by the fusion of nontransformed animal cells containing said desired genomic component with cells of a transformed cell line sensitive to predetermined medium conditions, which method comprises:

providing said desired genomic component with a marker that overcomes the sensitivity of the cells of the transformed cell line to the medium conditions;

mixing said nontransformed animal cells with cells of said transformed cell line under conditions that promote fusion to obtain a fusion mixture;

selecting for fused cell colonies by culturing said fusion mixture under said predetermined medium conditions; and

screening successful colonies for the presence of said desired genomic component.

2. The method of claim 1 wherein the marker is HPRT and the medium conditions comprise HAT medium.

3. The method of claim 1 wherein the marker is the neomycin resistance gene and the medium conditions comprise the presence of G418.

4. The method of claim 1 wherein said desired genomic component is a pair of immunoglobulin loci, one

for heavy and one for light chain, and the nontransformed cell is a B cell

5. The method of claim 4 wherein the pair of immunoglobulin loci is expressed to produce an antibody immunoreactive with a target antigen.

6. The method of claim 1 wherein the transformed cell line is a myeloma cell line.

7. The method of claim 1 wherein said conditions that promote fusion comprise the presence of polyethylene glycol.

8. An improvement in a method to obtain colonies of fused cells that contain a desired genomic component, which fused cells are obtained by the fusion of nontransformed animal cells containing said desired genomic component with cells of a transformed cell line sensitive to predetermined medium conditions and wherein said method comprises:

mixing said nontransformed animal cells with cells of said transformed cell line under conditions that promote fusion to obtain a fusion mixture;

selecting for fused cell colonies by culturing said fusion mixture under said predetermined medium conditions; and

screening successful colonies for the presence of said desired genomic component;

wherein said improvement comprises providing said desired genomic component with a marker that

overcomes the sensitivity of the cells of the transformed cell line to the medium conditions.

9. The improvement of claim 8 wherein the marker is HPRT and the medium conditions comprise HAT medium.

10. The improvement of claim 8 wherein the marker is the neomycin resistance gene and the medium conditions comprise the presence of G418.

11. The improvement of claim 8 wherein said desired genomic component is a pair of immunoglobulin loci, one for heavy and one for light chain and the nontransformed cell is a B cell.

12. The improvement of claim 11 wherein the pair of immunoglobulin loci is expressed to produce an antibody immunoreactive with a target antigen.

13. The improvement of claim 8 wherein the transformed cell line is a myeloma cell line.

14. The improvement of claim 8 wherein said conditions that promote fusion comprise the presence of polyethylene glycol.

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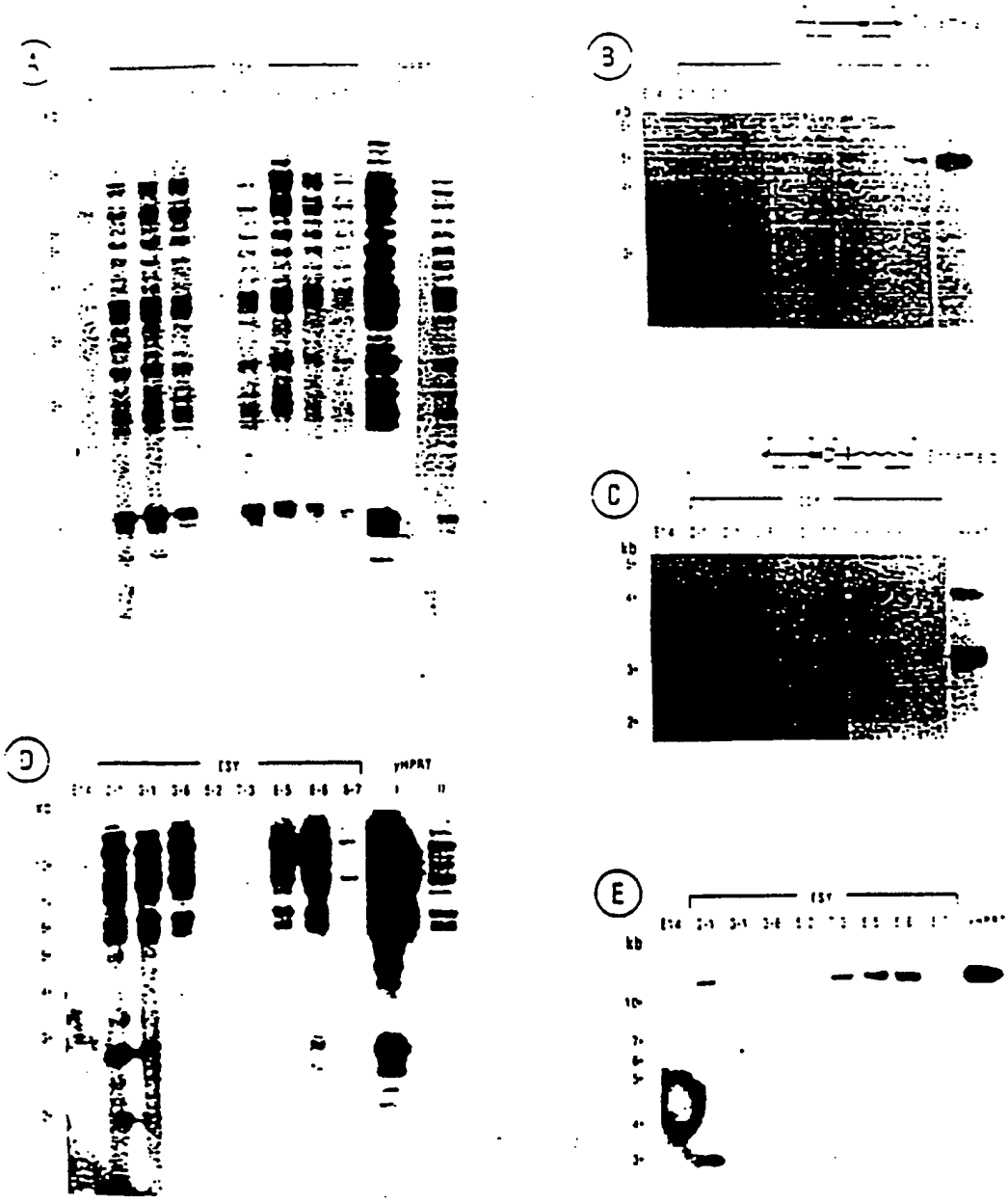


Figure 1

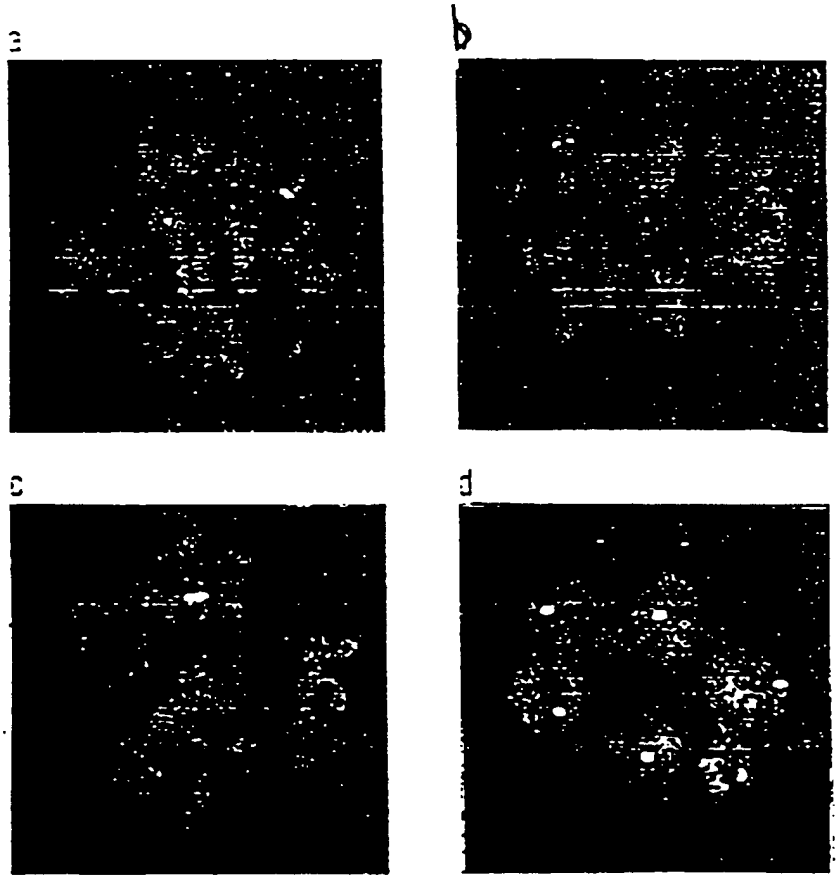
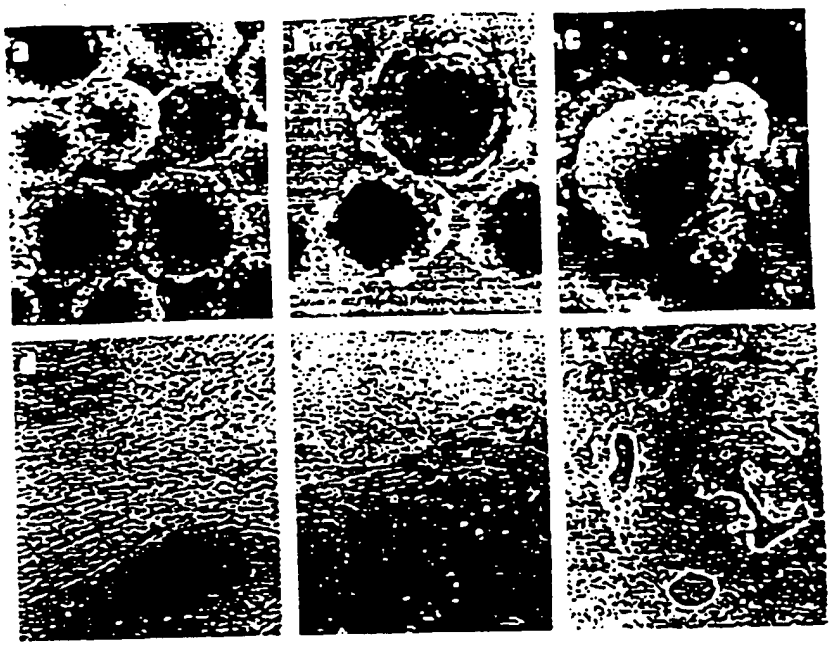


Figure 2



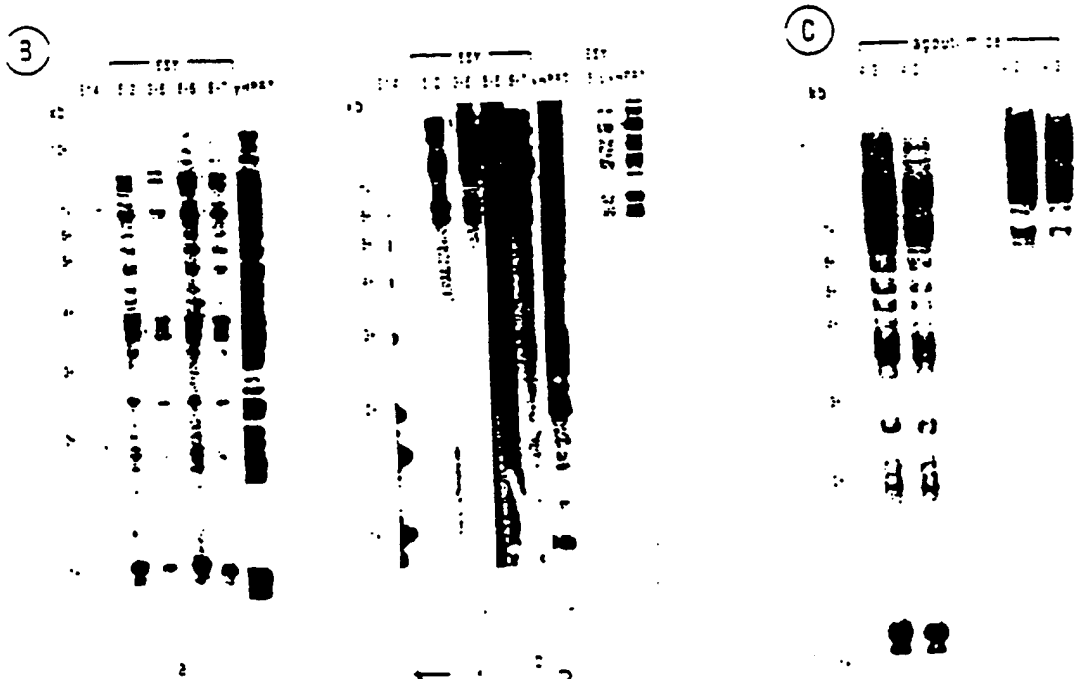


Figure 3

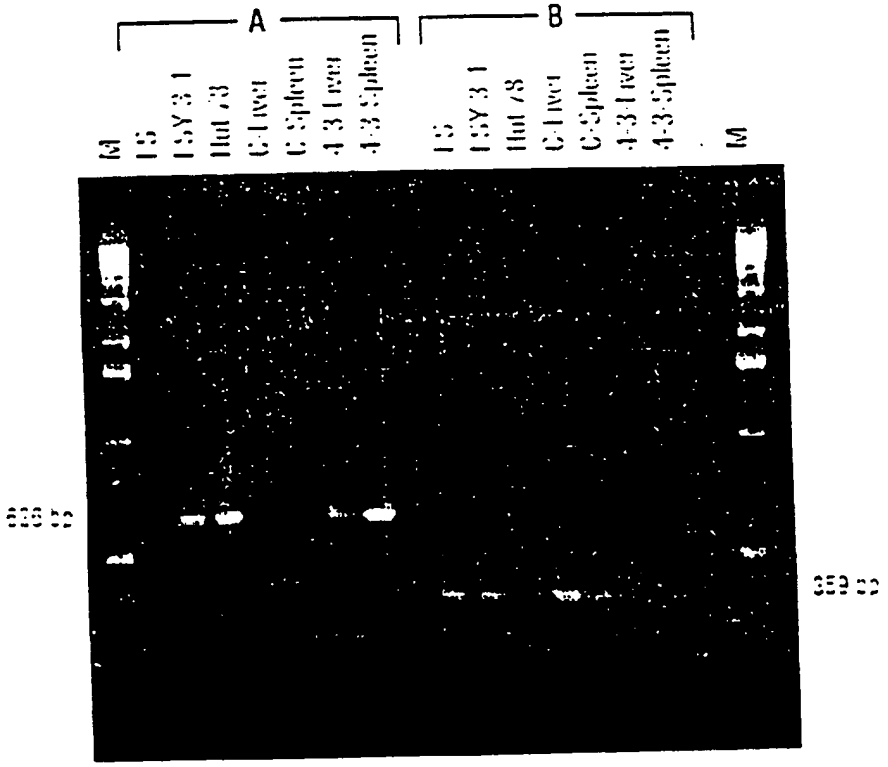


Figure 4

Mouse Breeding Scheme

Cross IA.

heterozygous inactive Murine IgH
X
heterozygous inactive Murine IgK

MIgH (inactive) MIgK
MIgH MIgK
X
MIgH MIgK (inactive)
MIgH MIgK

↓

F1 (cross I A)

MIgH (inactive) MIgK (inactive)
MIgH MIgK

Cross I B.

heterozygous Human IgH
X
heterozygous Human IgK

MIgH MIgK HIgH
MIgH MIgK
X
MIgH MIgK HIgK
MIgH MIgK

↓

F1 (cross I B)

MIgH MIgK HIgH HIgK
MIgH MIgK

Cross II. F1 (cross I A) x F1 (cross I B)

↓

F2 Quadruple Heterozygotes

MIgH (inactive) MIgK (inactive) HIgH HIgK
MIgH MIgK

Cross III. Intercross F2 mice

↓

F3 DOUBLE Homozygotes

MIgH (inactive) MIgK (inactive) HIgH HIgK
MIgH (inactive) MIgK (inactive)

Fig 5

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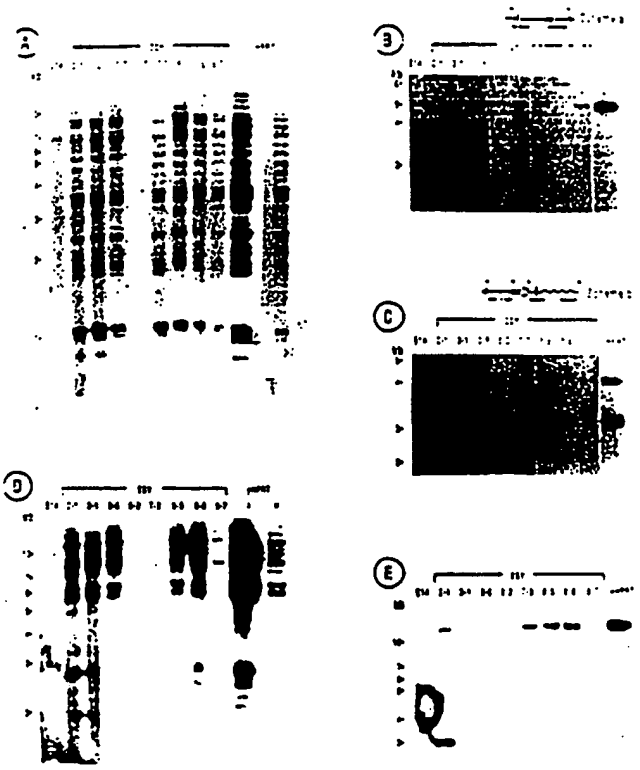
(54) Title: METHOD TO IMPROVE SCREENING EFFICIENCY IN FUSED CELLS

(57) Abstract

A method for improving the efficiency of screening fused cells for the presence of desired genes is disclosed. The method involves providing the desired gene with a marker that overcomes the sensitivity of an immortalizing cell fusion partner to certain medium conditions. The method is illustrated by the inclusion of a marker on immunoglobulin genes present in B cells.



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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 96/01169

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N5/16 C12N5/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 02602 (CELL GENESYS, INC.) 3 February 1994 cited in the application see page 6, line 21 - line 34 see page 10, line 27 - page 11, line 16 see page 12, line 24 - page 14, line 6 see page 16, line 31 - page 17, line 14 see page 18, line 21 - page 19, line 3 see page 20, line 15 - page 22, line 6 see page 62, line 5 - line 27; example IX --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 June 1996	18. 07. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, vol. 219, no. 4589, 11 March 1983, LANCASTER, PA US, pages 1228-1230, XP000573783 R. THOMAS TAGGART ET AL.: "Stable antibody-producing murine hybridomas" see abstract see page 1228, middle column, last paragraph - page 1229, left-hand column, paragraph 1 see page 1229, left-hand column, paragraph 3 - page 1230, left-hand column, paragraph 2	1-14
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A	EP,A,0 298 807 (TRANSGENE S.A.) 11 January 1989 see page 3, line 16 - line 26 see page 4, line 17 - line 22	1-14
A	EP,A,0 459 372 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 4 December 1991 see page 3, line 22 - line 28 see page 6, line 8 - line 11	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 96/01169

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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